

# *Salmonella*-Induced Mucosal Lectin RegIII $\beta$ Kills Competing Gut Microbiota

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## Abstract

Intestinal inflammation induces alterations of the gut microbiota and promotes overgrowth of the enteric pathogen *Salmonella enterica* by largely unknown mechanisms. Here, we identified a host factor involved in this process. Specifically, the C-type lectin RegIII $\beta$  is strongly upregulated during mucosal infection and released into the gut lumen. *In vitro*, RegIII $\beta$  kills diverse commensal gut bacteria but not *Salmonella enterica* subspecies I serovar Typhimurium (*S. Typhimurium*). Protection of the pathogen was attributable to its specific cell envelope structure. Co-infection experiments with an avirulent *S. Typhimurium* mutant and a RegIII $\beta$ -sensitive commensal *E. coli* strain demonstrated that feeding of RegIII $\beta$  was sufficient for suppressing commensals in the absence of all other changes inflicted by mucosal disease. These data suggest that RegIII $\beta$  production by the host can promote *S. Typhimurium* infection by eliminating inhibitory gut microbiota.

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## Introduction

Diarrhea is an infectious disease that causes high mortality worldwide, especially among children and the elderly [1]. *Salmonella* spp. infection is an important cause of gastroenteritis and has been widely studied because of facile *Salmonella* spp. genetics and excellent animal disease models [2,3,4]. Current treatment relies mostly on water and electrolyte supplementation as *Salmonellosis* is often self-limiting in humans [5]. Patients which show septic *Salmonellosis* are treated with antibiotics, but the rise of drug resistant *Salmonella* strains is a current problem in efficient *Salmonella* spp. therapy [5,6].

The normal intestinal microbiota of mammals counteracts infection by *Salmonella* spp. and other pathogens, a phenomenon referred to as colonization resistance (CR) [7,8,9,10]. The microbiota can impair *Salmonella* spp. by blocking adhesion sites, production of inhibitory or toxic molecules, or stimulation of the host's immune system [11,12,13,14]. However, intestinal inflammation can reduce CR by diminishing inhibitory microbiota and changing its composition. As a result, inflammation promotes high-level *Salmonella enterica* colonization and effective transmission by prolonged fecal shedding [15,16,17,18,19,20]. Interestingly, gut inflammation also enhances intestinal infection by other enteropathogenic bacteria such as *Citrobacter rodentium*, *Vibrio cholerae* or *Clostridium difficile* [21,22,23].

The mechanism promoting inflammation-mediated overgrowth of enteric pathogens is not fully understood. Inflammation could alter intestinal nutrient availability (i.e. mucus-derived carbohy-

drates) in a way that increases pathogen growth but diminishes resident microbiota ('nutrient hypothesis') [16,24]. Another reason for *S. Typhimurium* overgrowth in an inflamed gut is the production of tetrathionate from microbiota-derived H<sub>2</sub>S by the inflamed host mucosa [24]. Tetrathionate allows *Salmonella enterica* subspecies I serovar Typhimurium (*S. Typhimurium*) to perform a respiratory metabolism, which confers an advantage in the competition with the microbiota [24]. Alternatively, inflammation induces immune effector mechanisms that kill resident microbiota but not resistant pathogens ('killing hypothesis'). Indeed, the antimicrobial protein lipocalin-2, which is upregulated in inflamed mice and Rhesus macaques [25], blocks iron uptake in enteric bacteria by sequestering enterochelin. However, *S. Typhimurium* produces salmochelin, a glycosylated form of enterobactin, which is not bound by lipocalin-2 [26,27]. Hence, *S. Typhimurium* gains a selective advantage in the presence of lipocalin-2 [25]. These data suggest that there is not a single factor leading to *S. Typhimurium* outcompetition of the microbiota but more likely a whole range of parameters playing a role in inflammation.

Antimicrobial peptides (e.g., defensins, cathelicidins) constitute another host defence supporting the mucosal barrier. The C-type lectin RegIII $\gamma$ , a member of the Reg gene family, a diverse group of secreted proteins harboring a C-type lectin carbohydrate recognition domain, has been shown to exert antimicrobial effects on commensal bacteria [28]. The closely related RegIII $\beta$  has previously been shown to be upregulated in response to inflammation and infections [29,30]. Here, we studied the properties and *in vivo* relevance of the C-type lectin RegIII $\beta$  in

inflammation-induced microbiota-pathogen competition. This was of interest as RegIII family members are known to affect host-commensal and host-pathogen interactions in the intestine [28,30,31]. Our results imply that RegIII $\beta$  is indeed one of the host factors explaining why *S. Typhimurium* can benefit from triggering gut inflammation.

## Materials and Methods

### Ethics statement

All animals were handled in strict accordance with good animal practice and all animal work was approved by local animal care and use committees (license 04/862 Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit; license 201/2007 Kantonales Veterinäramt Zürich).

### Bacterial strains and growth conditions

All *S. Typhimurium* strains used in this study were derivatives of SL1344 *hisG rpsL xyl* [32] (TABLE 1). The virulent and avirulent *S. Typhimurium* strains have been described previously [33,34] as well as *S. Typhimurium*  $\Delta$ aroA [35]. *Salmonella* Typhimurium mutants with defined gene deletions were obtained using the Lambda phage red recombinase method [36] with primers described at <http://falkow.stanford.edu/whatwedo/wanner/> (see also TABLE 2). For comparison of LPS defect mutants, *Salmonella* Typhimurium LT *galE* [37] was used.

Non-pathogenic *Escherichia coli* E2 (*stx1*– *stx2*– *eaeA*– *hlyA*– *espP*– *katP*– *astA*– *recA*+ *tolC*+) was isolated from BALB/c mice originally obtained from Charles River and maintained at the

Hannover Medical School Animal Facility for 12 weeks. Streptomycin- and rifampicin-resistant E2 derivatives were obtained by consecutive selection of spontaneous mutants on media containing rising antibiotic concentrations. *Bacillus subtilis* 168 was obtained from the *Bacillus* Genetic Stock Center. For detailed description of gut commensal strains see TABLE 1.

For mouse infections, bacterial strains were grown for 12 h at 37°C in Luria-Bertani (LB) broth (0.3 M NaCl) and subcultured for 4 h, as described [34].

### Purification of recombinant Proteins

A synthetic sequence optimized for expression of murine RegIII $\beta$  (ProteinID: NP\_035166.1) without signal peptide in *E. coli* was constructed (GENEART, Regensburg) and cloned into pET11a (Novagen, Bad Soden). Variant RegIII $\beta$  R135T was constructed using PCR mutagenesis and verified by sequencing. RegIII $\beta$  and the variant were expressed in *E. coli* BL21 (DE3) and purified essentially as described [38]. In brief, cells were induced with 0.1 mM IPTG for 3 h. Cells were harvested by centrifugation, washed with PBS, and frozen at –20°C. Cells were thawed on ice and resuspended in IB buffer (20 mM Tris–HCl, 10 mM EDTA, 1% Triton X-100, 0.1 mg/ml Lysozyme, pH 7.5). Cells were lysed by sonication (7×20 s pulses) and RegIII $\beta$  inclusion bodies were sedimented by centrifugation. The pellet was resuspended in 25 ml IB buffer and sonification/centrifugation was repeated three times to reduce contaminations by intact cells. Purified inclusion bodies were resuspended in denaturing buffer (7 M Guanidine–HCl, 0.15 M reduced glutathione, 2 mM EDTA, 0.1 M Tris–HCl, pH 8.0). Still insoluble material was removed by centrifugation and

**Table 1.** Strains used in this study.

Strain	Genetic information	Strain information	Growth condition	Reference	Figure
<i>S. Typhimurium</i> wt	Wild type SL1344	virulent <i>S. Typhimurium</i>	LB medium	[33]	Fig. 1, Fig. 2, Fig. S1
<i>S. Typhimurium</i> avir	$\Delta$ invG, <i>sseD::aphT</i>	avirulent <i>S. Typhimurium</i>	LB medium	[34]	Fig. 1, Fig. 5 Fig. S1
<i>S. Typhimurium</i> avir	<i>invG::Cm</i> , <i>ssrB::Km</i>	avirulent <i>S. Typhimurium</i>	LB medium	This study	Fig. 4
<i>S. Typhimurium</i> $\Delta$ phoP	<i>phoP::Km</i>		LB medium	This study	Fig. 3
<i>S. Typhimurium</i> $\Delta$ rfe	$\Delta$ aroA, <i>rfe::Km</i>		LB medium	This study	Fig. 3
<i>S. Typhimurium</i> $\Delta$ rfeBP	$\Delta$ aroA, <i>rfeBP::Km</i>		LB medium	This study	Fig. 3
<i>S. Typhimurium</i> $\Delta$ galE	$\Delta$ galE	LT2	LB medium	[37]	Fig. 3
<i>S. Typhimurium</i> $\Delta$ rfaG	$\Delta$ aroA, <i>rfaG::Km</i>		LB medium	This study	Fig. 3
<i>S. Typhimurium</i> $\Delta$ pagL	$\Delta$ aroA, <i>pagL::Km</i>		LB medium	This study	Fig. 3
<i>S. Typhimurium</i> $\Delta$ pagP	$\Delta$ aroA, <i>pagP::Km</i>		LB medium	This study	Fig. 3
<i>E. coli</i> E2		Mouse intestinal isolate, selected for spontaneous Rifampicin resistance	LB medium	This study	Fig. 2, Fig. 3
<i>E. coli</i> Nissle 1917		Probiotic <i>E. coli</i>	LB medium	[64]	Fig. 3B
<i>E. coli</i> CFT073		Uropathogenic <i>E. coli</i>	LB medium	[65]	Fig. 3B
<i>B. subtilis</i>		168		<i>Bacillus</i> Genetic Stock Center	Fig. 3A
<i>L. reuteri</i> RR <sup>Rif</sup>		Mouse intestinal isolate, selected for spontaneous Rifampicin resistance	MRS medium, grown anaerobically, 37°C	[15]	Fig. 3B
<i>L. murinus</i> RR <sup>Rif</sup>		Mouse intestinal isolate, selected for spontaneous Rifampicin resistance	MRS medium, grown anaerobically, 37°C	This study	Fig. 3B
<i>E. faecalis</i>		Mouse intestinal isolate	MRS medium, grown anaerobically, 37°C	This study	Fig. 3B
<i>C. butyricum</i> DSM10702		Mouse intestinal isolate	Wilkins Chalgren agar supplemented with 5% defibrillated sheep blood (Oxoid), grown anaerobically	DSMZ, Braunschweig, Germany	Fig. 3B

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**Table 2.** Primers used in this study.

Primer	Sequences
<i>rfbP::Km</i>	rfbP-P1 (60 nt) 5'-ATT TTA TTT ACA TTA TGC ACG GTC AGA GGG TGA GGA TTA AGT GTA GGC TGG AGC TGC TTC-3' rfbP-P4 (60 nt) 5'-GCT AAT TTA TAC AAT TAT TAT TCA GTA CTT CTC GGT AAG CAT TCC GGG GAT CCG TCG ACC-3' rfbP-F (20 nt) 5'-ACC TGA GTT ACG CTG CTA TG-3' rfbP-R (19 nt) 5'-TCC TGT CAG GTG TGG AAA C-3'
<i>rfaG::Km</i>	rfaG-P1 (60 nt) 5'-TGC CGC ATG AGG CAC GCA CCA TAG ATT TGG ACA GCC TGC TGT GTA GGC TGG AGC TGC TTC-3' rfaG-P4 (60 nt) 5'-ATC TTT ACC GCG CCA TAG TGT GGT TAA CGG CGC TTT CAG CAT TCC GGG GAT CCG TCG ACC-3' rfaG-F (19 nt) 5'-CCG GCT GAA GAT GTT ATC G-3' rfaG-R (20 nt) 5'-GCG TCT CCA GCT CTC TGA AC-3'
<i>pagL::Km</i>	pagL-P1 (60 nt) 5'-GCC GGT TAA AAA TAA CTA TTG ACA TTG AAA TGG TGG TGG AGT GTA GGC TGG AGC TGC TTC-3' pagL-P4 (60 nt) 5'-TTA CTC CTT CAG CCA GCA ACT CGC TAA TTG TTA TTC AAC TAT TCC GGG GAT CCG TCG ACC-3' pagL-F (20 nt) 5'-TGC TAT ATC AGC CGT TTC TG-3' pagL-R (20 nt) 5'-CTG ATT GGA CAT CTT TCC TG-3'
<i>pagP::Km</i>	pagP-P1 (60 nt) 5'-GGT TAA TGT TGT TAT TAT CAC AGT CGA ATT TTT GAA CGG TGT GTA GGC TGG AGC TGC TTC-3' pagP-P4 (60 nt) 5'-TAA GAC TTT TTA ATT CAC AAC TGA AGC ATA CCC TTC CCC AAT TCC GGG GAT CCG TCG ACC-3' pagP-F (20 nt) 5'-ACG CCG TTA ACC CGA TAC TC-3' pagP-R (20 nt) 5'-ACG TCT TTG CTG CCA TCT TC-3'

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the supernatant was slowly diluted into ice-cold refolding buffer (0.5 M arginine-HCl, 0.6 mM oxidized glutathione, 50 mM Tris-HCl, pH 8.0). After overnight incubation, insoluble material was removed by centrifugation and the supernatant was concentrated using ultrafiltration with a 10 kDa cut-off membrane (Millipore). The concentrate was dialyzed twice against binding buffer (25 mM MES-NaOH, 25 mM NaCl, pH 6.0). Insoluble material was removed by centrifugation. The final material was homogenous as judged by SDS-PAGE. We verified by omitting lysozyme in the purification protocol that it doesn't contribute to bacterial killing in the *in vitro* assays.

For some experiments, RegIII $\beta$  was trypsinated with 500 ng/ml Trypsin (5000 NF-U mg<sup>-1</sup>) at a concentration of 50  $\mu$ g ml<sup>-1</sup> in binding buffer at 37°C for 2 h. For fluorescence detection, RegIII $\beta$  was covalently linked to Alexa647 (Molecular Probes) following the manufacturer's instructions.

Purification of RegIII $\gamma$  has been performed as described previously [38].

### Animal experiments

Mice were purchased from Charles River (Sulzfeld, Germany) or bred at the RCHCI ETH Zurich, Switzerland. Eight to 12 weeks old female BALB/c or C57BL/6 mice were pre-treated with 20 mg streptomycin and 24 h later intragastrically infected with  $5 \times 10^7$  CFU of *S. Typhimurium*, as previously described [39].

For some experiments, streptomycin-pretreated MyD88<sup>-/-</sup> and MyD88<sup>+/-</sup> mice (C57BL/6 background [40]) were infected with wild-type *S. Typhimurium* and sacrificed 24 h later.

Colonization was determined by plating feces on selective media (MacConkey agar with appropriate antibiotics). In contrast to many non-culturable commensals, the gut luminal cfu data of *S. Typhimurium* (and *E. coli*) obtained by plating always match the cfu data obtained in feces. This is well documented by data from plating, 16S RT PCR, in situ hybridization and immunofluorescence microscopy [9,15,39,60].

To determine the effect of RegIII $\beta$  on bacterial colonization, mice received a single intragastric dose, or daily doses of 80  $\mu$ g RegIII $\beta$  or 80  $\mu$ g BSA respectively, in 200  $\mu$ l binding buffer.

### In vitro assays

Binding and cidal activities of RegIII $\beta$  were determined using bacteria from late log liquid cultures. Bacteria were washed and

resuspended in binding buffer (25 mM MES-NaOH, 25 mM NaCl, pH 6.0) at a density of  $10^6$  CFU ml<sup>-1</sup>. RegIII $\beta$  was added at various concentrations and the mixture was incubated for various times at 37°C. Bacteria were then plated either on LB, Wilkins Chalgren, or MRS agar, and grown under aerobic or anaerobic conditions (7% H<sub>2</sub>, 10% CO<sub>2</sub>, 83% N<sub>2</sub>). As controls (= 100% survival) Bacteria incubated with binding buffer only, were analyzed.

In some experiments, RegIII $\beta$  was pre-incubated for 10 min with a fivefold (weight/weight) excess of peptidoglycan (insoluble PGN from *Bacillus subtilis*, Sigma) before addition to bacterial suspensions. Peptidoglycan co-precipitation was determined as described [28].

Bacteria were incubated for 40 min at 4°C with 1  $\mu$ M fluorescence-labeled RegIII $\beta$  in binding buffer containing 1% bovine serum albumin to minimize unspecific binding. At this low concentration, no cidal effect of RegIII $\beta$  that could potentially affect the results has been detected. Bacteria were washed three times in binding buffer and analyzed using a Calibur flow cytometer (BD Biosciences).

### Antibody generation, WB, and immunohistochemistry

A polyclonal rabbit anti-RegIII $\beta$  antibody was produced by Neosystem (Strasbourg, France) using recombinant RegIII $\beta$ . The antibody was further affinity purified with AminoLink Kit (Thermo Scientific Pierce) using recombinant RegIII $\beta$ . The antibody is specific for RegIII $\beta$  (Fig. S1).

For analysis of intestinal RegIII $\beta$ , cecal contents were resuspended in PBS. Proteins were separated by SDS-PAGE followed by Western blotting using the polyclonal anti-RegIII $\beta$  antibody and chemoluminescent detection (GE Healthcare).

For immunohistochemistry, cecal tissue samples were fixed overnight in PBS with 4% paraformaldehyde at 4°C, incubated in 20% sucrose at 4°C for one day, and snap frozen in O.C.T. compound (Sakura). Cryosections (10  $\mu$ m) were air dried, permeabilized with 0.1% Triton in PBS, blocked (10% goat serum in PBS), and stained with rabbit anti-RegIII $\beta$  antibody and cy3-labelled anti-rabbit antibody (Jackson). No tissue staining was observed with secondary antibody only. Nuclei were visualized with DAPI. The actin brush border was stained with Phalloidin-Alexa 647 (Fluoprobes). Images were taken with a Axiovert 200 (Carl Zeiss, Inc.) microscope, an Ultraview confocal head

(PerkinElmer), and a krypton argon laser (643-RYB-A01; Melles Griot). Infrared, red and green fluorescence was recorded confocally, and blue fluorescence was recorded by epifluorescence microscopy.

### Quantitative RT-PCR

RegIII $\beta$  mRNA levels were quantified using QIAGEN isolation kits, M-MLV reverse Transcriptase RNase H Minus, the QuantiTect SYBR Green PCR kit (Qiagen), and primers as described below.  $c_t$  values were normalized to GAPDH [41] and represent the median of triplicate analyses compared to non-infected mice. Cycling parameters were 94°C (15 s), 60°C (30 s), 72°C (30 s) in a RotorGene 3000 cycler (Corbett Research, Cambridgeshire, UK). The following Primers were used: GAPDH (5'-GGC TGC CCA GAA CAT CAT CCC TGC AT-3' and 5'-ACG TCA GAT CCA CGA CGG ACA CAT TGG-3') and RegIII $\beta$  (5'-CTG CCT TAG ACC GTG CTT TC-3' and 5'-ATA GGG CAA CTT CAC CTC AC-3').

### Statistical analysis

Statistical analysis was performed using the Student T-Test except for long-term colonization where the Mann Whitney U-test was applied.  $P \leq 0.05$  was considered to be statistically significant.

## Results

### *S. Typhimurium* infection induces intestinal RegIII $\beta$ expression

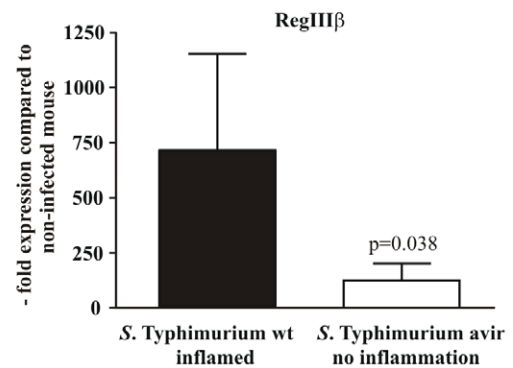
The C-type lectin RegIII $\beta$  is highly induced in rat intestinal tissues during enteric *Salmonellosis* [42,43]. We aimed at analyzing the role of RegIII $\beta$  in microbiota-pathogen competition in the streptomycin-treated mouse model for *S. Typhimurium* induced gut inflammation. To initially confirm validity of previous data for this model, we performed quantitative real time PCR of RegIII $\beta$  mRNA on cecal tissue (Fig. 1A). Mice infected with an avirulent *S. Typhimurium* strain that fails to cause intestinal inflammation, show slightly upregulated RegIII $\beta$  mRNA levels compared to baseline levels in non-infected control mice. In contrast, mice infected with wild-type *S. Typhimurium* (wt) show drastically increased levels (~10-fold; Fig. 1A).

To confirm qPCR data on protein level, we analyzed intestinal contents in Western blots using an affinity-purified polyclonal antibody to RegIII $\beta$  (Fig. S2A). This antibody recognizes a major 15 kDa band and a weaker band of approx. 14 kDa, which likely correspond to RegIII $\beta$  and a proteolytically processed form (Fig. 1B). Proteolytic cleavage by trypsin has been reported for the closely related C-type lectin RegIII $\gamma$  [28]. As trypsin is present in intestinal contents, RegIII $\beta$  processing is likely to occur *in vivo*.

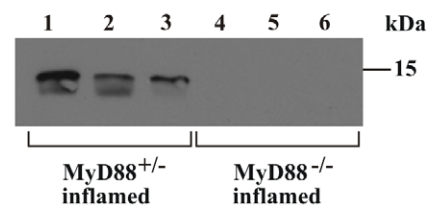
Quantification using purified recombinant RegIII $\beta$  as a reference indicated that RegIII $\beta$  was present at baseline levels of less than 1  $\mu\text{g/g}$  intestinal contents in uninfected mice, but increased to levels of some 350  $\mu\text{g/g}$  in mice infected with virulent *S. Typhimurium* (Fig. S2B).

*S. Typhimurium*-induced RegIII $\beta$  upregulation critically depended on the innate immunity adapter protein MyD88 (Fig. 1B) as previously reported in case of RegIII $\beta$  induction by the commensal microbiota [44]. The same is true for the closely related C-type lectin RegIII $\gamma$  in a *L. monocytogenes* infection model [30]. Immunohistochemistry of intestinal tissue sections revealed weak RegIII $\beta$  levels in *S. Typhimurium* infected mice, which were non-inflamed, but prominent RegIII $\beta$  expression in intestinal epithelial cells in mice with gut inflammation (Fig. 1C). These data confirmed and extended previous observations of RegIII $\beta$  upregulation in intestinal tissues in response to virulent *Salmonella* spp..

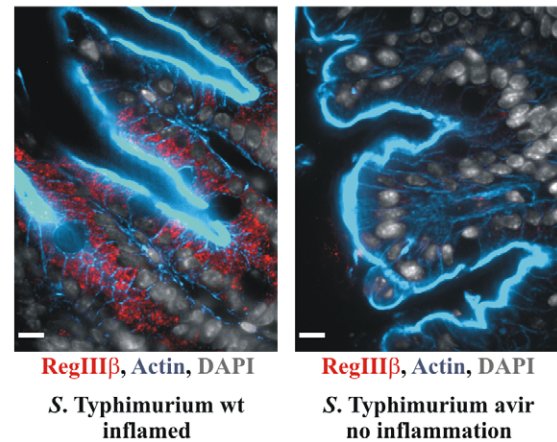
**A**



**B**



**C**



**Figure 1. *S. Typhimurium*-induced RegIII $\beta$  expression in mouse intestine.** A) RegIII $\beta$  expression was analyzed in the cecum of streptomycin-treated C57BL/6 mice infected for 24 h with *S. Typhimurium* wt and *S. Typhimurium* avir, respectively. Expression was analyzed by quantitative real time PCR normalized to GAPDH mRNA levels (fold expression versus unmanipulated mouse). B) Western Blot of intestinal contents obtained from *S. Typhimurium* infected MyD88<sup>+/+</sup> and MyD88<sup>-/-</sup> litter mates 24 h post infection using a polyclonal antibody to RegIII $\beta$ . Samples 1–3 were obtained from infected MyD88<sup>+/+</sup> mice and samples 4–6 from MyD88<sup>-/-</sup> litter mates. C) Immunohistochemistry of the cecal mucosa of *S. Typhimurium* infected mice 24 h post infection (grey, nuclei (DAPI); blue, actin (phalloidin); red, RegIII $\beta$ ). Scale bar: 10  $\mu\text{m}$ . doi:10.1371/journal.pone.0020749.g001

### RegIII $\beta$ kills commensal bacteria but not *S. Typhimurium*

The C-type lectin RegIII $\gamma$  has been reported to have bactericidal effects. To monitor the antimicrobial activity of closely related RegIII $\beta$  on different bacterial species, we produced recombinant RegIII $\beta$ . We tested its antibacterial spectrum in *in vitro* killing assays



against *S. Typhimurium* wt, *E. coli* and *B. subtilis*. *B. subtilis* and *E. coli* were chosen as these strains were used in other studies assessing bacterial susceptibility to RegIII $\gamma$  [28]. Since *Bacillus* spp. is not a normal member of the GI tract, several other Gram<sup>+</sup> bacteria typical for the GI tract were also included in the analysis (*Lactobacillus* spp., *Enterococcus* spp., *Clostridium* spp.). Aliquots of bacterial suspensions were incubated with recombinant RegIII $\beta$  and bacterial survival was analyzed after 30 minutes. The cfu of the negative control (no RegIII $\beta$ ) was taken as 100% survival rate. Interestingly, recombinant purified RegIII $\beta$  had bactericidal activity against *E. coli* but not *S. Typhimurium* and *B. subtilis*. We tested an extended panel of commensal gut bacteria but found no clear killing preference for Gram<sup>+</sup> vs. Gram<sup>-</sup> species (Fig. 2A, B). The related intestinal C-type lectin, RegIII $\gamma$  is known to selectively kill Gram<sup>+</sup> bacteria [28]. These data suggested that the two lectins may have complementary antibacterial profiles. Effective doses for bactericidal activity were below 2.5  $\mu$ M (Fig. 2A, Fig. S2B), which is in the range of the RegIII $\beta$  concentrations observed in the *S. Typhimurium*-infected intestine (see above). Since *S. Typhimurium* was found to be resistant to RegIII $\beta$ , we hypothesized that *S. Typhimurium*-induced RegIII $\beta$  could thus affect some commensal microorganisms in the intestinal lumen upon gut inflammation, and thereby contribute to the substantial ecological changes in gut microbiota and observed pathogen overgrowth during *S. Typhimurium* wt infection [15,19,45].

### Molecular structure correlates with bactericidal activity

To identify the mechanisms of *S. Typhimurium* resistance to RegIII $\beta$  we decided to determine the molecular target of RegIII $\beta$  on the bacterial cell envelope. An excess of peptidoglycan (PGN) completely blocked RegIII $\beta$  bactericidal activity (data not shown) consistent with the previously reported role of PGN as RegIII $\beta$  target [46]. Although RegIII $\beta$  did bind to bacteria containing various PGN types, including *S. Typhimurium* *in vivo* (data not shown), bactericidal activity was preferentially directed against particular PGN types. Specifically, some sensitive bacteria carried the negatively charged amino acid residue diaminopimelic acid at position 3 of the PGN pentapeptide (PGN-DAP: *E. coli*, *Clostridium butyricum*), whereas most resistant bacteria carried a neutral or positively charged amino acid at this position (*B. subtilis*, amidated diaminopimelic acid; *Lactobacillus casei*, *L. murinus* and *Enterococcus faecalis*, lysine) [47,48,49]. Interestingly, loop 2 of RegIII $\beta$ , which is homologous to the usual C-type lectin binding site but apparently not involved in binding the carbohydrate portion of peptidoglycan [46], contains a positively charged arginine (R135), while the equivalent residue is a threonine in RegIII $\gamma$  which kills bacteria with PGN-Lys. In an unrelated insect peptidoglycan binding protein PGRP-LE a similar arginine - threonine substitution controls selective activity against PGN-DAP vs. PGN-LYS [50]. To test the hypothesis that arginine 135 of RegIII $\beta$  promotes selective cidal interaction with negatively charged PGN, we generated a RegIII $\beta$  R135T variant by site-directed mutagenesis. RegIII $\beta$  R135T had weaker cidal activity against *E. coli* but gained cidal activity against *B. subtilis* that was lacking in the wild-type protein (Fig. 2C) supporting the role of amino acid 135 in selective interaction with certain PGN types. The still moderate activity of RegIII $\beta$  R135T against *B. subtilis* suggested multiple additional interactions as observed for other PGN-binding proteins [51]. This was also evident from wild-type RegIII $\beta$  resistance of *Lactobacillus reuteri* despite its Lys-type peptidoglycan.

### The O-antigen protects *S. Typhimurium* against RegIII $\beta$ killing

RegIII $\beta$  did not kill PGN-DAP containing *S. Typhimurium* but did kill *E. coli* containing the same type of PGN (Fig. 2A). This observation was consistent with the uncompromised viability of

*Salmonella enterica* isolated from infected gut contents [52]. As one possible resistance mechanism, *Salmonella* spp. outer membrane lipopolysaccharides (LPS) [53] could restrict access of RegIII $\beta$  to its target peptidoglycan in the periplasm. To test this hypothesis, we compared isogenic *S. Typhimurium* mutants with defined defects in LPS biosynthesis. These mutants lacked the O-antigen polymerase (*rfc*), the complete O-antigen (*rfbP*), the outer core and the O-antigen (*galE*) and the inner core, outer core and the O-antigen (*rfaG*) and certain lipid-A modifications (*phoP*, *pagL* and *pagP*) (Fig. 3C). Shortening of the polysaccharide component of LPS (Fig. 3A) progressively increased *S. Typhimurium* susceptibility to RegIII $\beta$ -mediated killing (Fig. 3B) as observed for other antimicrobial molecules [54]. Moreover, PhoP-dependent expression of *pagP* (involved in hepta-acetylation of lipid A with palmitate [55]) but not *pagL* (catalyzes the 3-O-deacylation of lipid A at position 3 [56]) was essential for *S. Typhimurium* resistance to RegIII $\beta$  (Fig. 3B). These data suggested that in wild-type *S. Typhimurium* carrying a rigid LPS layer, RegIII $\beta$  had limited access to peptidoglycan and therefore weak anti-*S. Typhimurium* activity. Interestingly, *E. coli* has similar LPS modification capabilities, but under our experimental *in vitro* conditions, these mechanisms were apparently insufficient to mediate resistance against RegIII $\beta$  in various *E. coli* strains.

### RegIII $\beta$ selectively suppresses *E. coli* in a simple co-colonization model

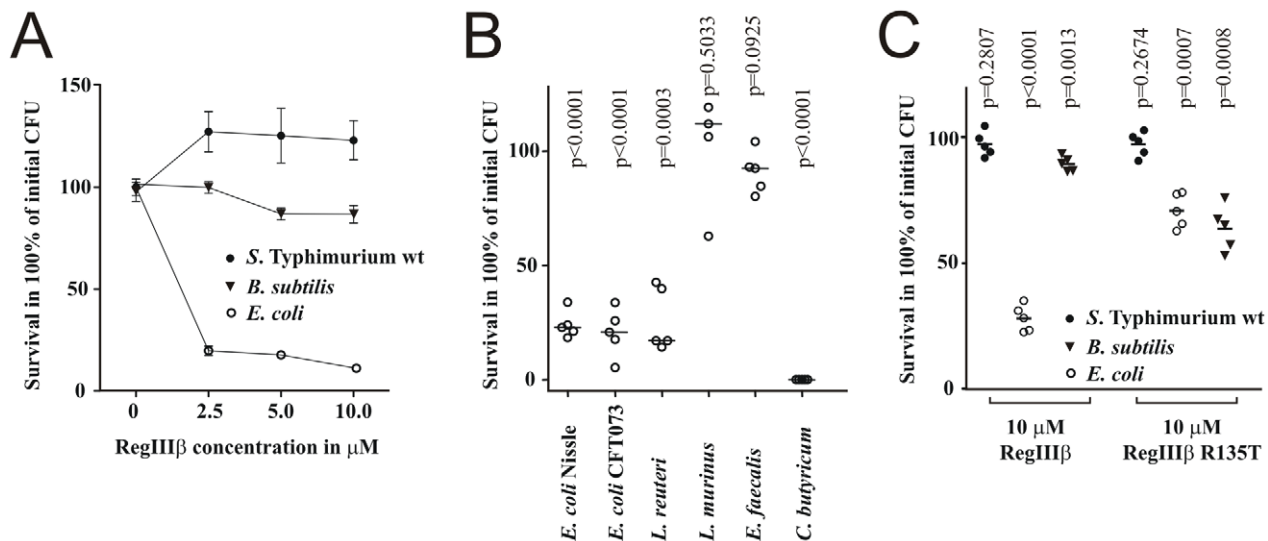
The *in vitro* data suggested that RegIII $\beta$  killed various bacterial species but not *S. Typhimurium*. *S. Typhimurium*-induced RegIII $\beta$  could thus contribute to ecological changes in gut microbiota during *S. Typhimurium* infection [15,19,45]. To test this hypothesis, we established a simple experimental mouse model employing intestinal colonization of streptomycin-pretreated mice [39] with *S. Typhimurium* and a molecularly defined (see Material & Methods sections) non-pathogenic, streptomycin-resistant *Escherichia coli* strain (*E. coli* E2) from our mouse colony. *S. Typhimurium* colonization preferentially occurs in individuals with high initial *E. coli* abundance [9], which suggests potential relevance for *S. Typhimurium*/*E. coli* competition.

When given individually, both *E. coli* E2 and *S. Typhimurium* colonized the mouse intestine at high levels in streptomycin-treated mice (data not shown). Virulent *S. Typhimurium* induced intestinal inflammation as previously reported, while an avirulent *S. Typhimurium* *invG* *ssrB* strain as well as the *E. coli* E2 isolate did not induce obvious pathology (data not shown) in agreement with previous reports [15,39,57,58,59]. Interestingly, virulent *S. Typhimurium* wt suppressed co-administered *E. coli* E2 (Fig. 4A), while avirulent *S. Typhimurium* permitted stable high level co-colonization of both species (Fig. 4B). Our simple co-colonization model thus reproduced important aspects of *S. Typhimurium*-induced commensal suppression.

To test whether the host factor RegIII $\beta$  might be sufficient for *E. coli* E2 *in vivo* suppression, we administered recombinant RegIII $\beta$  to mice co-colonized by *E. coli* E2 and avirulent *S. Typhimurium*. A single dose of 80  $\mu$ g RegIII $\beta$  (similar to endogenous RegIII $\beta$  levels induced by virulent *S. Typhimurium* wt, see above) administered at 5 h post infection rapidly suppressed *E. coli* but did not affect *S. Typhimurium* gut colonization (Fig. 4C). RegIII $\beta$  was thus sufficient for suppressing commensal *E. coli* E2 even in the absence of all other mechanisms that might further enhance this effect in the inflamed gut.

### RegIII $\beta$ supports *S. Typhimurium* infection in the presence of complex gut microbiota

A complex gut microbiota strongly inhibits *S. Typhimurium* gut colonization [9,15]. Through induction of RegIII $\beta$  (Fig. 1A), *S.*



**Figure 2. RegIII $\beta$  has bactericidal activity against several Gram<sup>+</sup> and Gram<sup>-</sup> intestinal bacteria.** A) Survival of *S. Typhimurium* (filled circles), *E. coli* (open circles), and *Bacillus subtilis* (triangles) after 30 min incubation with various concentrations of RegIII $\beta$ . Means  $\pm$  SD of five independent replicates are shown. B) Survival of various intestinal commensal bacteria after incubation with 10  $\mu$ M RegIII $\beta$  (1, *E. coli* Nissle; 2, *E. coli* CFT073; 3, *Lactobacillus reuteri*; 4, *Lactobacillus murinus*; 5, *Enterococcus faecalis*; 6, *Clostridium butyricum*). C) Bactericidal activities of 10  $\mu$ M RegIII $\beta$  and 10  $\mu$ M RegIII $\beta$  R135T with altered putative binding site against *S. Typhimurium*, *E. coli* and *B. subtilis* (symbols as in A). Statistical significances for deviation from results obtained for bacteria incubated with buffer only are shown. doi:10.1371/journal.pone.0020749.g002

*Typhimurium* might exploit the host to suppress competing microbiota. To test this hypothesis, the simple *E. coli*/*S. Typhimurium* co-colonization model in streptomycin-pretreated mice was inappropriate. The competing microbiota is already weakened in this model. Consequently, even avirulent *S. Typhimurium* can efficiently colonize for several days even in the absence of intestinal inflammation.

To test if RegIII $\beta$ -induction could offer *S. Typhimurium* any benefit under more natural conditions, we tested conventional mice containing a normal gut microbiota (no streptomycin pretreatment). We determined the fecal density of facultative aerobic bacteria in our mouse colony by plating (Fig. 5A). This technique closely reflects the gut luminal bacterial counts based on previous data from plating, 16S RT PCR, in situ hybridization and immunofluorescence microscopy [9,15,39,60]. However, it is important to notice that luminal (or fecal) counts do not necessarily correlate with mucosal colonization levels. One group of mice was orally treated with a single dose of BSA, the other group with recombinant RegIII $\beta$ , both followed by an oral dose of *S. Typhimurium* ( $5 \times 10^7$  cfu, by gavage). BSA-treated mice did not show efficient *S. Typhimurium* luminal colonization (Fig. 5A) as observed previously [9,39,61]. However, oral treatment with a single dose of RegIII $\beta$  suppressed endogenous Gram<sup>-</sup> facultative aerobic microbiota and enabled subsequent *S. Typhimurium* luminal colonization at moderate levels (Fig. 5A).

In another model, we followed long-term shedding of *S. Typhimurium* in streptomycin-pretreated mice during recolonization with competing microbiota, which terminates pathogen growth in the gut after approximately 4 days, in the absence of mucosal inflammation [15,60]. Due to its defect to trigger inflammation, the avirulent *S. Typhimurium* strain is overgrown by the competing commensal flora. We aimed at testing if oral supplementation of RegIII $\beta$  could interfere with reemerging microbiota-induced colonization resistance. Indeed, daily RegIII $\beta$  administration significantly prolonged *S. Typhimurium* fecal shedding (Fig. 5B). The combined data showed that RegIII $\beta$  can

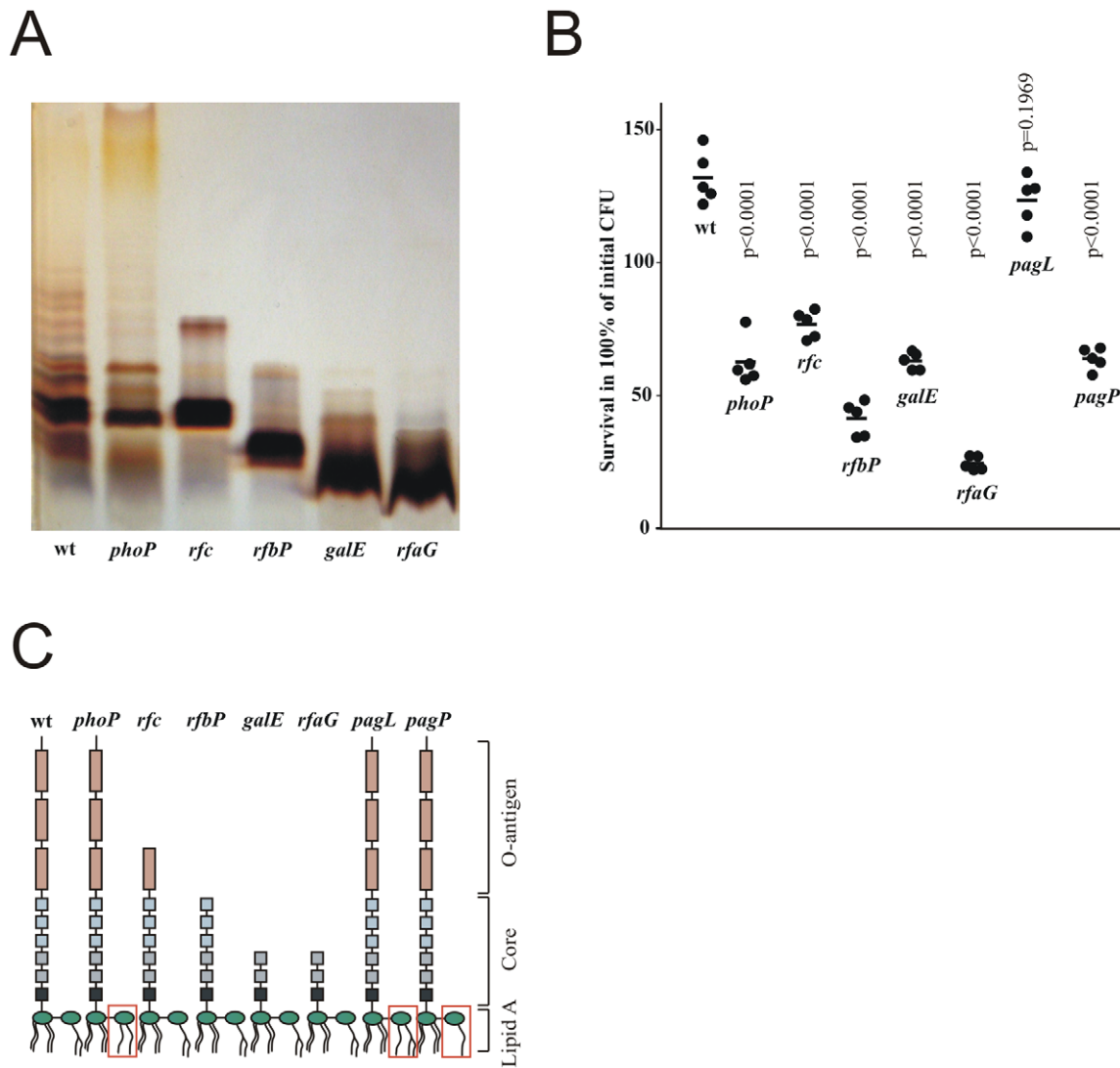
enhance *S. Typhimurium* gut luminal colonization in the presence of complex commensal microbiota.

## Discussion

*S. Typhimurium* induces a complex inflammatory response in the intestinal mucosa. This inflammation has a profound impact on gut microbiota composition. Among others, microbiota that competes with *S. Typhimurium* is suppressed whereas *S. Typhimurium* colonization is promoted. The molecular mechanisms of this process are probably complex and poorly understood [10]. On one side, altered nutrient availability in the inflamed gut may lead to positive selection of the pathogen over the commensal flora [16,24]. On the other side numerous host defense factors are differentially regulated during intestinal inflammation [25,42,43, 62] and could mediate selective killing of the commensal microbiota [17,20]. Their relevance for facilitating *S. Typhimurium* colonization has remained unclear.

Among the host factors induced by *S. Typhimurium*, the C-type lectin RegIII $\beta$  is particularly strongly upregulated. RegIII $\beta$  can block mucosal infections with pathogenic *Yersinia* spp. although it does not inhibit *Yersinia* spp. directly [31]. RegIII $\beta$  is also highly related to another C-type lectin, RegIII $\gamma$  that kills Gram<sup>+</sup> bacteria [28] and protects against mucosal *Listeria* infections [30]. For these reasons, we studied the role of RegIII $\beta$  in enteric salmonellosis.

We showed that *S. Typhimurium* infection induced elevated RegIII $\beta$  protein levels in the intestine. Recombinant RegIII $\beta$  bound peptidoglycan *in vitro* and killed a diverse set of both Gram<sup>-</sup> and Gram<sup>+</sup> bacteria but not *S. Typhimurium*. Site-directed mutagenesis of RegIII $\beta$  and comparison of various *S. Typhimurium* mutants suggested that peptidoglycan structure and lipopolysaccharide composition (for Gram<sup>-</sup> bacteria) might explain the bactericidal activity spectrum of RegIII $\beta$ . Recently published work points out that HIP/PAP (hepatointestinal pancreatic/pancreatitis associated protein), a human ortholog of RegIII $\gamma$  interacts with the



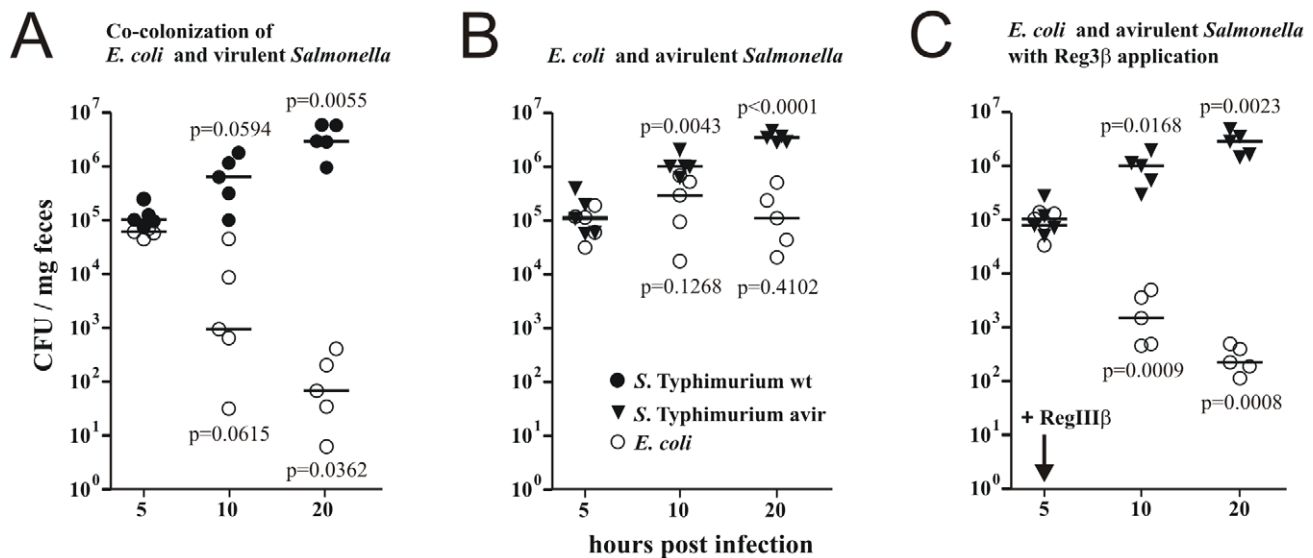
**Figure 3. The O-antigen mediates *S. Typhimurium* resistance against RegIII $\beta$ .** A) SDS-PAGE (silver stain) of lipopolysaccharide of wild-type *S. Typhimurium* (lane 1) and mutants (*phoP*, *rfc*, *rfbP*, *galE*, *rfaG*). B) Bactericidal activity of 10  $\mu$ M RegIII $\beta$  against various *S. Typhimurium* mutants. Statistical significances for deviation from results obtained for wildtype bacteria are shown. C) Schematic representation of *Salmonella* spp. LPS-forms. doi:10.1371/journal.pone.0020749.g003

carbohydrate moiety of peptidoglycan which may be also the case for RegIII $\beta$  [46]. The observed antibacterial spectrum of RegIII $\beta$  was clearly different from the reported spectrum of the closely related RegIII $\gamma$  suggesting that these two mucosal lectins might serve complementary functions in modulation of gut microbiota. We hypothesize that *S. Typhimurium* is protected against RegIII $\beta$  dependent killing by specific modifications mediating a rigid LPS layer. Possibly, differences between *S. Typhimurium* and *E. coli* in PhoP-dependent regulatory circuits [63] and/or subtle differences in LPS structure might affect the susceptibility for RegIII $\beta$ . Future work will have to address the structural mechanisms underlying differential RegIII $\beta$  killing of *E. coli* and *S. Typhimurium*.

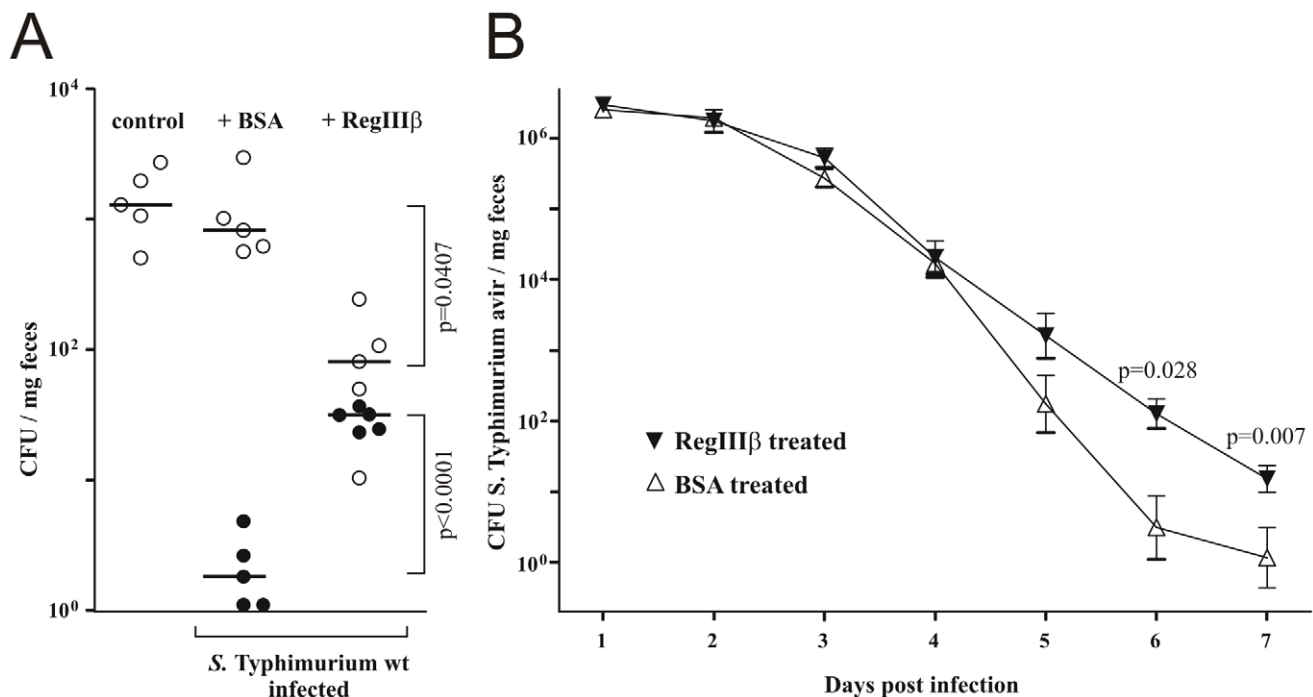
Doses in the range of endogenous RegIII $\beta$  levels were orally administered and could efficiently suppress *E. coli* gut colonization and facilitate *S. Typhimurium* colonization. Our findings suggest that RegIII $\beta$  may contribute to inflammation-induced suppression of competing microbiota although we have no direct evidence of

RegIII $\beta$  playing a role *in vivo*. Experiments using RegIII $\beta$  ko mice may aid clarifying this issue.

Presently, we do not know the exact effects of RegIII $\beta$  treatment on microbiota composition and function. This might be an important topic for future research. However, effects resulting from RegIII $\beta$  feeding were moderate compared to the much stronger facilitation of *S. Typhimurium* colonization in mice with acute intestinal inflammation. Thus, additional host factors and bacterial fitness factors apparently also contribute significantly to inflammation-induced microbiota changes and pathogen overgrowth. Possible candidates for these additional factors include the paralogue RegIII $\gamma$  with a complementary bactericidal spectrum, defensins and other yet unidentified, *Salmonella*-encoded fitness factors. Further work will be required to elucidate the respective relevance and the unknown mechanisms, and how they might be integrated with the function of the previously identified host and bacterial factors in the microbiota-pathogen interplay in the inflamed intestine.



**Figure 4. RegIII $\beta$  is sufficient to suppress *E. coli* during competitive growth in the non-inflamed gut.** A) Colonization of streptomycin-pretreated mice with virulent *S. Typhimurium* wt (filled circles) and non-pathogenic *E. coli* E2 (open circles). Virulent *S. Typhimurium* induces gut inflammation and suppresses co-colonization with *E. coli*. B) Avirulent *S. Typhimurium* permits *E. coli* co-colonization. C) A single dose of RegIII $\beta$  is sufficient to complement the suppression defect of avirulent *S. Typhimurium*. Each data point represents one mouse. Statistical significances for deviation from results obtained at 5 h are shown. doi:10.1371/journal.pone.0020749.g004



**Figure 5. RegIII $\beta$  facilitates *S. Typhimurium* colonization in mice with complex microbiota.** A) Colonization of mice with normal intestinal microbiota (no streptomycin pre-treatment) with facultative aerobes (open circles, colonies on MacConkey agar plates; left). Colonization levels of facultative aerobes after a single oral dose of BSA or RegIII $\beta$  and subsequent infection with *S. Typhimurium* (closed circles; middle and right). A single dose of RegIII $\beta$  suppressed resident facultative aerobic bacteria and partially relieved colonization resistance against *S. Typhimurium*. Statistical significances for deviation from results obtained with BSA alone are shown. B) RegIII $\beta$  facilitates prolonged gut colonization by avirulent *S. Typhimurium* in streptomycin-pretreated mice. Daily RegIII $\beta$  doses (80  $\mu$ g in 200  $\mu$ l) prolonged *S. Typhimurium* colonization (filled down triangles) compared to control mice that received bovine serum albumin (open up triangles). In panel A) one data point represents one mouse; in panel B) one data point represents the median with SD of 5 mice. Statistical significances for deviation from results obtained for BSA alone are shown. Comparable data were obtained in an independent experiment. doi:10.1371/journal.pone.0020749.g005



## Supporting Information

**Figure S1 RegIII $\beta$  levels in intestinal content of C57BL/6 mice.** A) Western Blot of mouse intestinal contents with a purified polyclonal antiserum to RegIII $\beta$ . Samples 1–3 were obtained from streptomycin-treated mice infected with *S. Typhimurium* avir whereas samples 4–6 were taken from mice infected with virulent *S. Typhimurium* 1 day post infection. B) Western Blot showing different dilutions of recombinant RegIII $\beta$  compared to intestinal content (lane 6) of mouse infected with virulent *S. Typhimurium*. (lane 1: 0.552  $\mu$ g; lane 2: 0.276  $\mu$ g; lane 3: 0.138  $\mu$ g; lane 4: 0.069  $\mu$ g; lane 5: 0.0345  $\mu$ g; lane 6: 0.26 mg of intestinal content). Lane 6 is equivalent to 92 ng of RegIII $\beta$

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